EXHIBIT 5

Development 127, 4147-4156 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV3227

Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference

Petr Svoboda*, Paula Stein*, Harutoshi Hayashi and Richard M. Schultz‡

Department of Biology, University of Pennsylvania, Philadelphia, PA 19104-6018, USA
*These authors contributed equally to this work
‡Author for correspondence (e-mail: rschultz@mail.sas.upenn.edu)

Accepted 17 July; published on WWW 7 September 2000

SUMMARY

Specific mRNA degradation mediated by double-stranded RNA (dsRNA), which is termed RNA interference (RNAi), is a useful tool with which to study gene function in several systems. We report here that in mouse oocytes, RNAi provides a suitable and robust approach to study the function of dormant maternal mRNAs. *Mos* (originally known as c-*mos*) and tissue plasminogen activator (tPA, *Plat*) mRNAs are dormant maternal mRNAs that are recruited during oocyte maturation; translation of *Mos* mRNA results in the activation of MAP kinase. dsRNA directed towards *Mos* or *Plat* mRNAs in mouse oocytes effectively results in the specific reduction of the targeted mRNA in both a time- and concentration-dependent manner. Moreover, dsRNA is more potent than either sense or antisense RNAs. Targeting the *Mos* mRNA results in inhibiting the appearance of MAP kinase activity and can result in parthenogenetic activation. *Mos* dsRNA, therefore, faithfully phenocopies the *Mos* null mutant. Targeting the *Plat* mRNA with *Plat* dsRNA results in inhibiting production of tPA activity. Finally, effective reduction of the *Mos* and *Plat* mRNA is observed with stoichiometric amounts of *Mos* and *Plat* dsRNA, respectively.

Key words: Maternal mRNA, RNA interference, mouse oocyte, *Mos*, *Plat*

INTRODUCTION

'Omne vivum ex ovo' (All living things come from eggs), which is attributed to William Harvey, is probably the first articulation of the current view that the program for early development is established during oogenesis. During oogenesis in the mouse, oocytes grow and acquire the ability to resume and complete meiosis (acquisition of meiotic competence) (Sorensen and Wassarman, 1976; Wickramasinghe et al., 1991), as well as the ability to be fertilized and develop to term (acquisition of developmental competence) (Eppig and O'Brien, 1996). Meiotic maturation and egg activation are accompanied by the recruitment of many maternal mRNAs (Schultz et al., 1979; Schultz and Wassarman, 1977; Van Blerkom, 1981), and presumably some of these direct the synthesis of proteins that are required for the formation of a fertilizable egg that is capable of developing to term. One such mRNA is the *Mos* mRNA. The mobilization of the *Mos* mRNA results in the ultimate activation of mitogen-activated protein (MAP) kinase, whose activity is required to maintain arrest at metaphase II (Gebauer and Richter, 1997; Sagata, 1997); oocytes lacking the *Mos* gene mature to metaphase II but then undergo spontaneous activation, i.e., they emit the second polar body and form a pronucleus (Colledge et al., 1994; Hasimoto et al., 1994). The tissue plasminogen activator (tPA, *Plat*) mRNA is another maternal mRNA that is recruited during oocyte maturation (Huarte et al., 1987; Vassalli et al., 1989). Although tPA is synthesized during maturation and secreted following fertilization, and then becomes associated with a cell-surface receptor on the embryo (Carroll et al., 1993), *Plat* knockout mice are viable and fertile, but do display mild perturbations in phenotype, e.g., retardation in neuronal migration (Seeds et al., 1999).

To date, an antisense RNA approach has been the most widely used method to assess the function of maternal mRNAs that are recruited during oocyte maturation. Nevertheless, this approach has problems. For example, an antisense RNA approach has been used to assess the role of *Mos* mRNA recruitment during maturation. The phenotypes observed range from permitting germinal vesicle breakdown but inhibiting emission of the first polar body (Paules et al., 1989; Zhao et al., 1991), to emission of the first polar body but entering interphase instead of proceeding to and arresting at metaphase II (O'Keefe et al., 1989). In contrast, the phenotype of a *Mos* null mutant generated by homologous recombination is that the oocytes proceed to metaphase II, but meiotic arrest is not maintained and the eggs spontaneously undergo parthenogenetic activation (Colledge et al., 1994; Hasimoto et al., 1994). This discrepancy between the phenotypes observed by the antisense approach with that of a 'true' knockout potentially confounds the use of antisense RNA to study the function of a dormant maternal mRNA. Antisense RNA can

also target and destroy the *Plat* mRNA. The efficacy of destruction of the untranslated *Plat* mRNA, however, appears restricted to antisense RNA directed towards the 3′ UTR (Strickland et al., 1988). Antisense RNAs directed at other portions of the *Plat* mRNA are far less effective and can form hybrids only following maturation and the concomitant recruitment of the *Plat* mRNA. Thus, the efficacy of this approach is compromised by the appropriate selection of the region of the mRNA to be targeted, and this can only be determined experimentally and not a priori.

Recently, RNA interference (RNAi), which employs double-stranded RNA (dsRNA), has been shown to ablate potently the targeted mRNA in a variety of species (Sanchez-Alvardado and Newmark, 1999; Fire et al., 1998; Kennerdell and Carthew, 1998; Li et al., 2000; Lohmann et al., 1999; Misquitta and Paterson, 1999; Ngo et al., 1998; Wargelius et al., 1999). The destruction of the targeted mRNA by dsRNA occurs prior to translation (Fire, 1999; Montgomery et al., 1998; Sharp, 1999; Zamore et al., 2000), and targets exon sequences; dsRNA directed against intron sequences is ineffective (Fire et al., 1998). Genetic approaches in *Caenorhabditis elegans* have identified genes with homology to eIF-2C, RNase D, and RNA-directed RNA polymerase (Ketting et al., 1999; Tabara et al., 1999; Smardon et al., 2000) that are involved in the RNAi-mediated pathway of mRNA degradation. Very recent studies suggest that a nuclease involved in the destruction of the targeted mRNA contains an essential RNA component containing approx. 25-nucleotide RNAs that are homologous to the dsRNA (Hammond et al., 2000). The processing of the dsRNA to these fragments does not require the presence of the targeted mRNA, and the targeted mRNA is cleaved only in the regions of identity to the dsRNA and at sites that are 21-23 nucleotides apart (Zamore et al., 2000).

We report here that dsRNA directed towards *Mos* and *Plat* mRNAs in mouse oocytes effectively results in the specific reduction of the targeted mRNA in both a time- and concentration-dependent manner. Moreover, dsRNA is more potent than either sense or antisense RNA. Targeting the *Mos* mRNA results in inhibiting the appearance of MAP kinase activity, as well as promoting parthenogenetic activation of the treated cells, and targeting *Plat* mRNA results in inhibiting production of tPA activity. Effective reduction of the *Mos* and *Plat* mRNA is observed with stoichiometric amounts of *Mos* and *Plat* dsRNA, respectively. While these studies were in progress, a paper appeared that has reported that oocytes injected with *Mos* dsRNA undergo egg activation, as evidenced by pronucleus formation (Wianny and Zernicka-Goetz, 2000).

MATERIALS AND METHODS

dsRNA preparation

For *Mos* amplification, a pair of primers was designed based on the cDNA sequence (Accession number J00372). The sequence of upstream *Mos* primer was 5′-CCATCAAGCAAGTAAACAAG-3′ and the downstream *Mos* primer was 5′-AGGGTGATTCCAAAAGA-GTA-3′. These primers generated a PCR product that was 535 bp in length and corresponded to the 3′ end of the coding region and the beginning of the 3′UTR. Likewise, for *Plat* amplification a pair of primers was designed based on the cDNA sequence (Accession number J03520). The sequence of the upstream *Plat* primer was 5′-CATGGGCAAGCGTTACACAG-3′ and the downstream *Plat* primer was 5′-CAGAGAAGAATGGAGACGAT-3′. These primers generated a PCR product that was 650 bp in length and corresponded to the middle part of the coding region.

To generate template for transcription in vitro, 5 μg of liver total RNA were reverse transcribed with Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's instructions using oligo-dT as the primer. PCR amplification conditions for both *Mos* and *Plat* were as follows: initial denaturation at 94°C for 4 minutes was followed by 36 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute and the final cycle had an extended incubation at 72°C for 7 minutes followed by decrease to 4°C. All PCR reactions were performed in either a PE2400 or PE9600 PCR thermocycler.

Gel-purified primary PCR products were diluted 1:500 and re-amplified to produce specific templates to generate sense and antisense transcripts by transcription in vitro. To do this, primers were made that contained an SP6 promoter attached to the 5′ end of both the forward and reverse primers. Following PCR under the above conditions, the secondary PCR products were purified using a Nucleospin Extraction Kit (Clontech). The template (500-1000 ng) was then transcribed with SP6 RNA polymerase (Promega) in order to obtain the corresponding sense and antisense RNAs.

The in vitro transcription products were resolved following electrophoresis in 1.5% NuSieve LM agarose (FMC, Rockland, ME, USA) and the bands corresponding to the sense and antisense single-stranded RNA were purified according to the manufacturer's protocol. Equimolar amounts of sense and antisense RNA were then annealed in 1 mM Tris-HCl (pH 7.5), containing 1 mM EDTA, or in DEPC-treated water supplemented with 5% RNasin (Promega); similar results were obtained using either procedure. Typically, 2-4 μg of RNA in 30 μl were mixed and heated in 500 ml of boiling water for 1 minute. The sample, still in the water bath, was allowed to cool to room temperature over the course of several hours. The dsRNA was phenol extracted, ethanol precipitated, washed in 75% ethanol and then dissolved in water. Samples were stored in water at −80°C prior to use.

RNA isolation and RT-PCR

RNA was isolated from oocytes and prepared for RT-PCR as previously described (Temeles et al., 1994). In each case, 0.125 pg of rabbit β-globin mRNA/oocyte was added prior to RNA isolation. The globin mRNA serves as an internal standard for the efficiency of the RT-PCR reactions (Temeles et al., 1994). For each set of gene-specific primers the linear region of semi-log plots of the amount of PCR product as a function of cycle number was determined and a cycle number for each primer pair was selected that was in this linear range; the amount of PCR product under these conditions is proportional to the number of cells used (Manejwala et al., 1991). This method permits the comparison of relative changes in the abundance of a particular transcript (Ho et al., 1995; Latham et al., 1994; Temeles et al., 1994).

Following reverse transcription two oocyte equivalents were used as a template for each PCR reaction. PCR products were labeled with [α-^{32}P]dCTP (Amersham, 0.25 μCi per 50 μl reaction). PCR amplification conditions for both *Mos* and *Plat* were as follows: initial denaturation at 94°C for 2 minutes was followed by 28 (*Plat*) or 31 (*Mos*) cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, followed by 4°C until the samples were removed. PCR amplification conditions for globin: initial denaturation at 94°C for 2 minutes was followed by 24 cycles of 94°C for 10 seconds and 62°C for 15 seconds followed by final 4°C. After PCR, the products were subjected to electrophoresis in an 8% polyacrylamide gel. The gel was dried under vacuum for 1 hour at 80°C, exposed in phosphorimager cassette for 4 to 24 hours and the signal was quantified using the Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Oocyte collection, microinjection and culture

Fully grown, germinal vesicle (GV)-intact oocytes were obtained from pregnant mare's serum gonadotropin (PMSG)-primed six-week-old female CF-1 mice (Harlan) and freed of attached cumulus cells, as previously described (Schultz et al., 1983). The collection medium was bicarbonate-free minimal essential medium (Earle's salt) supplemented with polyvinylpyrrolidone (3 mg/ml) and 25 mM Hepes, pH 7.3. Germinal vesicle breakdown was inhibited by including 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX). The oocytes were transferred into CZB medium (Chatot et al., 1989) containing 0.2 mM IBMX (CZB+IBMX) and cultured in an atmosphere of 5% CO_2 in air at 37°C. Oocytes were microinjected in bicarbonate-free CZB containing 10 mM Hepes and 0.2 mM IBMX with 5 pl of the corresponding solution; the injections were performed as previously described (Kurasawa et al., 1989). The concentration of the undiluted stock solution was 0.2 μg/μl and injection of 5 pl of either *Mos* or *Plat* dsRNA corresponds to 1.7×10^6 and 1.4×10^6 molecules, respectively. When single-stranded RNA was injected, it was diluted to a concentration such that injection of 5 pl corresponded to the same number of molecules as when dsRNA was injected. In experiments in which either enzyme activity or phenotype was assayed, microinjected oocytes were cultured in CZB+IBMX for 10 or 20 hours. They were then washed through ten drops of IBMX-free CZB and cultured in CZB until oocyte collection and lysis. In experiments in which mRNA levels were measured, the oocytes were kept in medium containing IBMX for 10, 20, or 40 hours until they were collected and processed for RNA isolation.

tPA assay

tPA activity was assayed by zymography of single oocytes. Immobilon-P (Millipore) was soaked in methanol for 1 minute and then rinsed four times with 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl. The wet membrane was placed on Whatman paper soaked with this buffer and both were transferred to a 96-well dot blot apparatus (Milliblot Systems, Millipore) with the Immobilon-P facing upwards. A 96-well template was then placed on the stage and single oocytes were transferred in 1-2 μl of CZB medium to the middle of where the wells would form. The apparatus was completely assembled and the wet membrane with the oocytes was then exposed to vacuum suction for 2 minutes. The apparatus was then disassembled and the membrane was immediately applied on the detection gel; the detection gel, which contained 40 μg/ml of plasminogen (Fluka), was prepared as previously described (Vassalli et al., 1984). Zymograms were developed for 12-64 hours at 37°C, scanned with a black background and the lysed area was estimated using the ImageQuant software (Molecular Dynamics).

Histone H1 and MBP kinase assay

The activities of both histone H1 and myelin basic protein (MBP) kinases were determined in single eggs as follows: single eggs were transferred in 1.5 μl of culture medium into a tube containing 3.5 μl of double kinase lysis buffer (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM *p*-nitrophenyl phosphate, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 5 mM EGTA). The tubes were immediately frozen on dry ice and stored at −80°C until the assay was performed. The kinase reaction was initiated by the addition of 5 μl of double kinase buffer (24 mM *p*-nitrophenyl phosphate, 90 mM β-glycerophosphate, 24 mM $MgCl_2$, 24 mM EGTA, 0.2 mM EDTA, 4.6 mM sodium orthovanadate, 4 mM NaF, 1.6 mM dithiothreitol, 60 μg/ml aprotinin, 60 μg/ml leupeptin, 2 mg/ml polyvinyl alcohol, 2.2 μM protein kinase A inhibitor peptide (Sigma), 40 mM 3-(n-morpholino) propanesulfonic acid (MOPS), pH 7.2, 0.6 mM ATP, 2 mg/ml histone (type III-S, Sigma), 0.5 mg/ml MBP) with 500 μCi/ml [γ-^{32}P]ATP (3000 Ci/mmol; Amersham). To determine the background level of phosphorylation for H1 and MBP, 5 μl of double kinase lysis buffer was added instead of the egg lysate. The reaction was conducted for 30 minutes at 30°C and terminated by the addition of 10 μl double-strength concentrated SDS-PAGE sample buffer (Laemmli, 1970) and boiling for 3 minutes. Following SDS-PAGE, the 15% gel was fixed in 10% acetic acid/30% methanol, dried and exposed to a phosphorimager screen for 16 to 24 hours. Scanning and quantification of the signal were performed using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). For each experiment, the mean value of H1 or MBP kinase activities for oocytes microinjected with sense *Mos* RNA was arbitrarily set as 100% and the values obtained in the other groups of eggs were expressed relative to this amount.

RESULTS

Preparation of dsRNA

dsRNA for either *Mos* or *Plat* was prepared by hybridizing equimolar amounts of gel-purified, single-stranded sense and antisense transcripts that were generated by transcription in vitro of appropriate templates containing an SP6 promoter. In each case, the RNA was directed towards a coding portion of the transcript. Following hybridization, the single-stranded RNAs were essentially totally converted to dsRNA, as evidenced by the absence of any visible staining in the region of the gel that corresponded to single-stranded species (Fig. 1). The quantitative nature of hybridization permitted use of the dsRNA without any need for gel purification of the dsRNA species.

Effect of *Mos* and *Plat* dsRNA on *Mos* and *Plat* mRNA levels in mouse oocytes

Mos and *Plat* are two maternal mRNAs that are recruited during oocyte maturation (see Introduction). We selected to target the *Mos* mRNA since a *Mos* null oocyte has a defined phenotype, i.e., the oocyte matures to metaphase II, but rather than arresting at metaphase II, it undergoes spontaneous egg activation. In addition, it is possible to measure MAP kinase activity in a single oocyte; MAP kinase activity reflects the mobilization of *Mos* mRNA (see below). We also selected to target *Plat* mRNA, which like *Mos*, is a moderately abundant mRNA; it has been estimated that an oocyte contains approx. 10,000 transcripts each of *Mos* and *Plat* (Huarte et al., 1987;

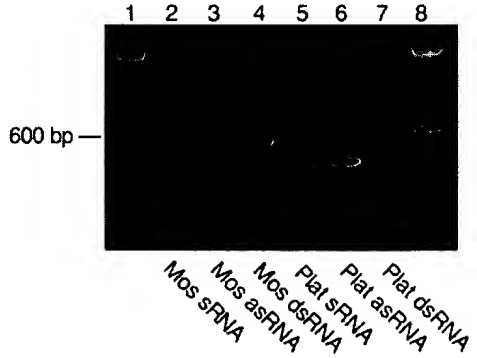

Fig. 1. Generation of *Mos* or *Plat* dsRNA. Sense or antisense *Mos* or *Plat* RNAs were produced by transcription in vitro and then gel purified. Equimolar concentrations of sense and antisense RNA were then hybridized and a portion of the reaction analyzed by electrophoresis. Shown is an ethidium bromide-stained gel demonstrating the quantitative formation of dsRNA. Lanes 1 and 8, 100 bp ladder.

Keshet et al., 1988). In addition, it is also possible to measure tPA activity in single oocytes.

Oocytes were injected with approx. 10^6 molecules of either *Plat* or *Mos* dsRNA that was directed towards the coding region of each transcript; this corresponds to approx. 10 nM final concentration (see Discussion). The oocytes were then cultured in medium containing IBMX to inhibit resumption of meiosis; a decrease in cAMP is associated with resumption of meiosis and including the membrane-permeable phosphodiesterase inhibitor IBMX in the medium prevents the decrease in cAMP and thus the resumption of meiosis (Schultz et al., 1983). Following culture, RNA was isolated and the relative amount of *Plat* and *Mos* transcripts were determined by a semi-quantitative RT-PCR assay that permits quantification of relative changes in transcript abundance. Prior to RNA isolation, a known amount of rabbit globin mRNA was added; this served as a control for RNA recovery, and for the efficiency of the RT-PCR (Temeles et al., 1994).

Oocytes injected with *Mos* dsRNA displayed a marked reduction in the amount of *Mos* transcript (approx. 80%), relative to water-injected or uninjected controls (Fig. 2, compare lane 2 with lanes 4 and 5). Likewise, oocytes injected with *Plat* dsRNA displayed an approx. 90% reduction in the amount of *Plat* transcript relative to the control (Fig. 2, lane 3). Specificity of this effect was demonstrated by the finding that *Mos* dsRNA did not reduce the abundance of *Plat* mRNA, and reciprocally, that *Plat* dsRNA did not reduce the abundance of *Mos* mRNA (Fig. 2). Results of these experiments indicated that the machinery for RNAi-mediated degradation of the targeted endogenous mRNA is present and functions in mouse oocytes.

Effect of *Mos* and *Plat* sense and antisense RNA on *Mos* and *Plat* mRNA levels in mouse oocytes

In other systems, antisense RNA can be ineffective. For example, injection of *C. elegans* with antisense RNA directed towards the *unc*-22 gene does not result in the mutant twitching phenotype, whereas dsRNA does (Fire et al., 1998). Nevertheless, antisense RNA can be effective in degrading oocyte mRNAs (Strickland et al., 1988). Accordingly, we determined the effect of sense and antisense *Mos* and *Plat* RNA on targeting the cognate oocyte transcript.

Oocytes were injected with approx. 10^6 copies of either sense, antisense or dsRNA, and incubated for 20 hours in medium containing IBMX, before the RNA was isolated and transcript abundance determined. As anticipated, dsRNA directed towards either *Mos* or *Plat* mRNA resulted in the reduction of the targeted mRNA, whereas the untargeted transcript remained essentially intact (Fig. 3A). As also anticipated, injection of sense RNA resulted in little, if any, decrease in the abundance of either the targeted or nontargeted mRNA. Injection of either *Mos* or *Plat* antisense RNA, however, did result in a decrease in the targeted, but not in the nontargeted, mRNA (Fig. 3A). Little, if any decrease in the targeted mRNA was observed when the amount of injected *Mos* or *Plat* antisense RNA was decreased by 10-fold (Fig. 3B). In contrast; this amount of dsRNA was effective in decreasing the amount of the targeted mRNA (Fig. 3B), e.g., the *Mos* dsRNA resulted in an approx. 85% decrease in *Mos* mRNA, and *Plat* dsRNA resulted in an approx. 30% decrease in *Plat* mRNA. Results of these experiments suggest that dsRNA is more effective in targeting mRNAs than antisense RNA.

Concentration- and time-dependence of dsRNA directed towards *Mos* and *Plat* mRNAs

In the experiments described above, the oocytes were injected

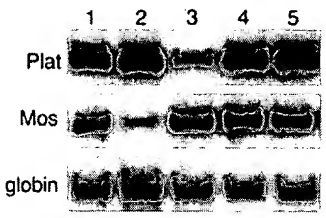

Fig. 2. Effect of *Mos* and *Plat* dsRNA on the relative abundance of *Mos* and *Plat* transcripts. Oocytes were injected with approx. 10^6 molecules of either *Mos* or *Plat* dsRNA and then cultured in the presence of IBMX for 20 hours. RNA was isolated and the relative amount of *Mos* and *Plat* transcripts were determined by RT-PCR, as described in Materials and Methods. The intensity of the globin band permits comparison of the different lanes, as it normalizes for RNA recovery, and for the efficiency of the RT-PCR part of the assay. Lane 1, relative amount of transcripts present in uninjected oocytes at t=0 hours; lane 2, relative amount of transcripts at t=20 hours in oocytes injected with *Mos* dsRNA; lane 3, relative amount of transcripts at t=20 hours in oocytes injected with *Plat* dsRNA; lane 4, relative amount of transcripts at t=20 hours in water-injected oocytes; lane 5, relative amount of transcripts at t=20 hours in uninjected oocytes.

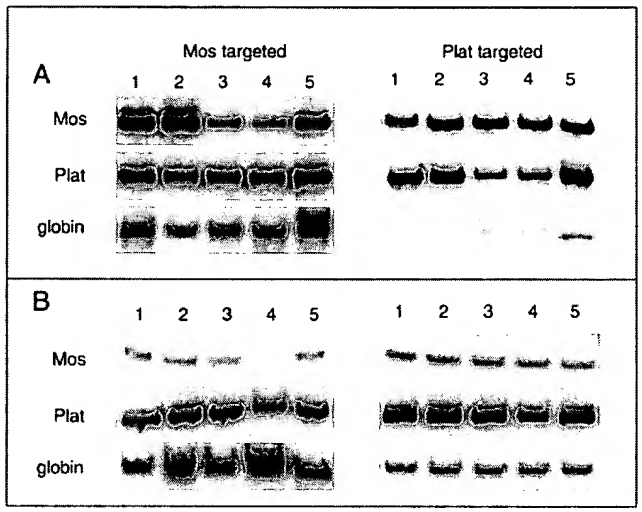

Fig. 3. Effect of *Mos* and *Plat* sense, antisense, and dsRNA on the relative abundance of *Mos* and *Plat* transcripts. (A) Oocytes were injected with approx. 10^6 molecules of either *Mos* or *Plat* sense, antisense or dsRNA and then cultured in the presence of IBMX for 20 hours. RNA was then isolated and the relative amount of *Mos* and *Plat* transcripts were determined by RT-PCR as described in the Materials and Methods. (B) Oocytes were injected with 10^5 molecules of either *Mos* or *Plat* sense, antisense or dsRNA and then processed as described in A. Lane 1, relative amount of transcripts present in water-injected oocytes; lane 2, relative amount of transcripts in sense RNA-injected oocytes; lane 3, relative amount of transcripts in antisense RNA-injected oocytes; lane 4, relative amount of transcripts in dsRNA-injected oocytes; lane 5, relative amount of transcripts in uninjected oocytes.

with approx. 10^6 molecules of dsRNA and cultured for 20 hours prior to determining the relative amount of targeted transcript. In order to determine further characteristics of the RNAi effect, the concentration- and time-dependence of this effect were determined. Oocytes were injected with 10^6, 10^4, or 10^2 molecules of either *Mos* or *Plat* dsRNA, and then incubated for 10, 20, or 40 hours prior to determining the relative abundance of the endogenous *Mos* and *Plat* transcripts (Fig. 4). For both *Mos* and *Plat* dsRNA-injected oocytes, the targeted message was destroyed in both a time- and concentration-dependent manner. In all cases, the nontargeted mRNA was not destroyed (data not shown).

Injection of 10^6 or 10^4 molecules of *Mos* dsRNA resulted in a substantial reduction in the amount of *Mos* mRNA, such that by 20 hours more than 75% of the mRNA was degraded; 10^2 molecules of injected *Mos* dsRNA had little, if any effect over the 40-hour timecourse. Although 10^6 molecules of injected *Plat* dsRNA also dramatically reduced the amount of *Plat* mRNA, the kinetics of *Plat* mRNA degradation were slower, when compared with those obtained for *Mos* dsRNA. In addition, 10^4 molecules of *Plat* dsRNA was not nearly effective as 10^4 molecules of *Mos* dsRNA. Similar to *Mos* dsRNA, the 100 molecules of injected *Plat* dsRNA was ineffective in reducing the amount of *Plat* mRNA.

Effect of Mos dsRNA on MAP kinase and p34^{cdc2}/cyclin B kinase activities

The experiments described above documented that both *Mos* and *Plat* dsRNA could result in the degradation of the targeted mRNA in a concentration- and time-dependent manner. We next demonstrated that the reduction of the targeted mRNA resulted in loss of formation of the encoded protein. The translation of *Mos* mRNA that initiates at the onset of oocyte maturation results in synthesis of MOS, which in turn activates MAP kinase kinase by phosphorylation (Gebauer and Richter, 1997; Sagata, 1997). MAP kinase kinase, which is a dual-specificity kinase, then phosphorylates MAP kinase on Thr183 and Tyr185 in the mammal, which in turn results in MAP kinase activation (Nishida and Gotoh, 1993). MAP kinase, which is a component of cytostatic factor (CSF) and is required to maintain metaphase II arrest, is frequently assayed by measuring the phoshorylation of MBP. Concomitant with germinal vesicle breakdown is the activation of p34^{cdc2}/cyclin B kinase (MPF) (Gebauer and Richter, 1997; Sagata, 1997), which is routinely assayed by phosphorylation of histone H1. In the mouse, MPF activation precedes MAP kinase activation by about 1-2 hours, and both activities reach maximal levels in the metaphase II-arrested egg (Verlhac et al., 1993). Following fertilization, MPF activity declines prior to MAP kinase activity (Moos et al., 1995; Verlhac et al., 1993).

Oocytes were injected with either *Mos* dsRNA, antisense RNA or sense RNA and cultured for 20 hours in IBMX-containing medium, then transferred to IBMX-free medium. The oocytes then matured to metaphase II, at which time both MAP and p34^{cdc2}/cyclin B kinase activities were assayed simultaneously in single eggs. As expected, sense RNA did not inhibit either kinase activity when compared with uninjected or water-injected eggs (data not shown). In contrast, both *Mos* antisense and dsRNA inhibited MAP kinase activity, although a greater degree of inhibition was observed with dsRNA (Fig. 5). This result was consistent with *Mos* dsRNA eliciting a greater decrease in *Mos* mRNA than *Mos* antisense RNA (Fig. 3). Although *Mos* antisense RNA did inhibit MAP kinase activity, the level of p34^{cdc2}/cyclin B kinase, i.e., histone H1 kinase, was reduced by only about 25% relative to control sense-injected or uninjected oocytes, while a 70% decrease was observed in the dsRNA-injected oocytes. This reduced amount of histone H1 kinase activity in the dsRNA-injected oocytes

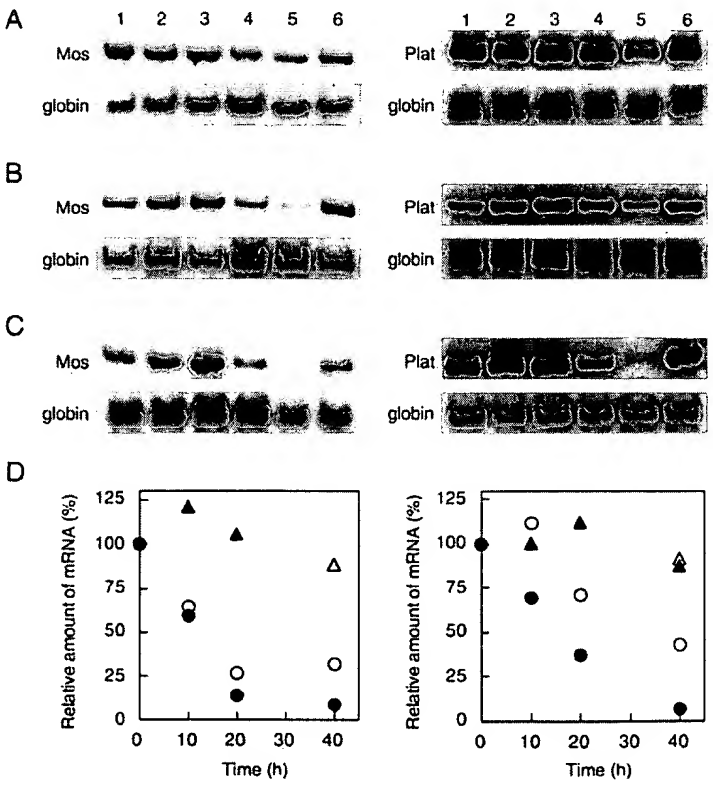

Fig. 4. Concentration- and time-dependence of *Mos* and *Plat* dsRNA-mediated reduction of the targeted mRNA. Oocytes were injected with 10^2 (lane 3), 10^4 (lane 4) or 10^6 (lane 5) molecules of either *Mos* or *Plat* dsRNA, and the relative abundance of either the *Mos* or *Plat* transcript was assayed after either 10 hours (A), 20 hours (B) or 40 hours (C) of culture in medium containing IBMX. Lane 1, relative amount of transcripts present in uninjected oocytes at t=0; lane 2, relative amount of transcripts in water-injected oocytes; lane 6, relative amount of transcripts in uninjected oocytes. (D) Quantification of the relative amount of *Mos* or *Plat* transcripts. The data are normalized to the amount present in the uninjected oocytes at the appropriate time following culture in IBMX-containing medium and all data are normalized to the globin signal, i.e., the ratio of the pixel volume of the transcript to that of the globin is set as 100%. (●), 10^6 molecules injected; (○), 10^4 molecules injected; (▲), 10^2 molecules injected; (△), the amount of *Plat* transcript present in oocytes injected with 10^6 molecules of *Mos* dsRNA or the amount of *Mos* transcript in oocytes injected with 10^6 molecules of *Plat* dsRNA. In order to keep the y-axis of similar scale, the value for oocytes injected with 10^2 molecules of *Mos* dsRNA and analyzed at 40 hours is not shown.

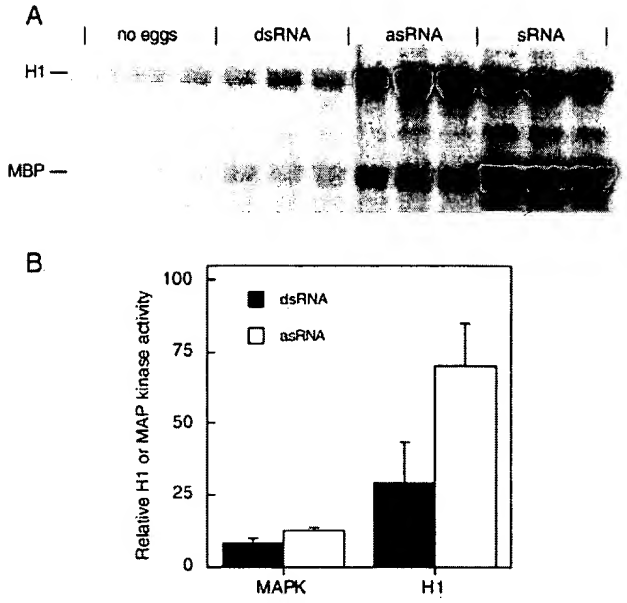

Fig. 5. Effect of *Mos* sense, antisense, and dsRNA on MAP kinase and MPF activities. Oocytes were injected with 10^6 molecules of either *Mos* sense (s), antisense (as) or dsRNA (ds), and then incubated for 20 hours in IBMX-containing medium. The oocytes were then transferred to IBMX-free medium and allowed to mature to metaphase II (about 18 hours), at which time single oocytes were assayed for both MAP kinase activity and MPF activity using MBP and histone H1, respectively as substrates. (A) Region of autoradiogram showing where phosphorylated histone H1 and MBP migrate. (B) Relative amount of kinase activity. The data have been normalized to that present in oocytes injected with sense RNA and this value does not differ from uninjected oocytes (data not shown). The data are expressed as the mean±s.e.m. and represent a total of 15, 15 and 13 dsRNA-, asRNA- and sRNA-injected oocytes, respectively.

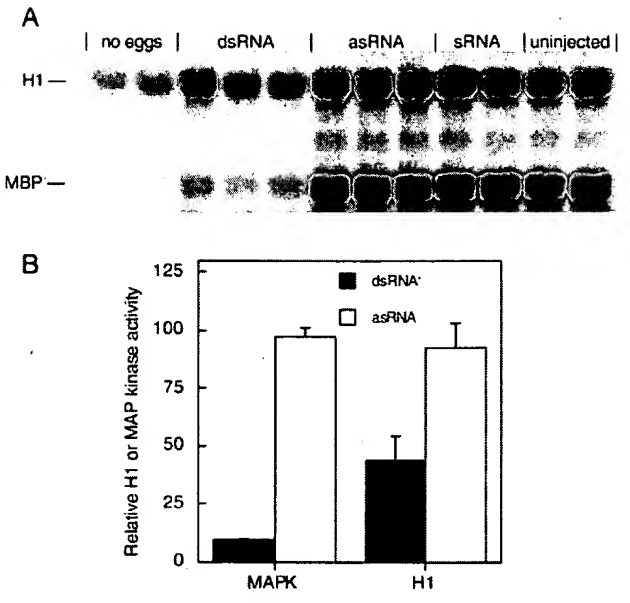

Fig. 6. Effect of *Mos* sense, antisense and dsRNA on MAP kinase and MPF activities. Oocytes were injected with 10^5 molecules of either *Mos* sense (s), antisense (as) or dsRNA (ds) and the experiment was then conducted as described in the legend to Fig. 5. (A) Region of autoradiogram showing where phosphorylated histone H1 and MBP migrate. (B) Relative amount of kinase activity. The data have been normalized to that present in oocytes injected with sense RNA and this value does not differ from uninjected oocytes. The data are expressed as the mean±s.e.m. and represent a total of 10, 10 and 6 dsRNA-, asRNA- and sRNA-injected oocytes.

was a consequence that in numerous cases these eggs underwent spontaneous egg activation, which results in a decrease in histone H1 kinase activity. In contrast, the antisense-injected oocytes never underwent egg activation.

Although *Mos* antisense RNA, which did result in a decrease in *Mos* mRNA, could inhibit MAP kinase activation, the results presented in Fig. 3 indicate that dsRNA is a more potent inhibitor than antisense RNA. Accordingly, oocytes were injected with 1/10 the amount of *Mos* sense, antisense, or dsRNA, cultured for 20 hours in IBMX-containing medium and then matured to metaphase II by transferring them to IBMX-free medium. The eggs were then assayed for both MAP and $p34^{cdc2}$/cyclin B kinase activities simultaneously in individual eggs. Whereas both *Mos* sense and antisense RNA did not inhibit the appearance of MAP kinase activity ($p34^{cdc2}$/cyclin B kinase activity was also high in these eggs), *Mos* dsRNA still elicited a dramatic inhibition in MAP kinase activity, and a corresponding decease in $p34^{cdc2}$/cyclin B kinase activity (Fig. 6). These results strengthen the conclusion that *Mos* dsRNA is more potent than *Mos* antisense RNA in promoting the reduction of the endogenous *Mos* mRNA.

Effect of Plat dsRNA on tPA activity

tPA is synthesized during oocyte maturation and its activity can be assayed in single oocytes by zymography (Huarte et al., 1985, 1987; Strickland et al., 1988). We observed only a single band (M_r 72,000) when metaphase II-arrested eggs were used; no activity was observed in GV-stage oocytes (data not shown). The presence of a single activity responsible for generating the lytic zone permitted analysis of tPA activity by simply spotting an oocyte/egg on a membrane, which was then overlaid with an agarose gel containing non-fat dry milk and plasminogen. The area of the lytic zone was linear as a function of time after a lag, which probably reflected the time to activate the zymogen cascade and degrade enough substrate to be visible to the eye (Fig. 7).

We assayed the effect of *Plat* sense, antisense and dsRNA on tPA activity in matured oocytes. Oocytes were injected with approx. 10^6 molecules of each RNA and cultured in IBMX-containing medium for 20 hours prior to initiating maturation by transfer to IBMX-free medium. Culture for 18 hours resulted in the production of metaphase II-arrested eggs that were then assayed for tPA activity. Injection of either antisense RNA or dsRNA resulted in a dramatic reduction in the amount of tPA activity, when compared with sense-injected oocytes (Fig. 8, black bars). The ability of *Plat* antisense RNA to inhibit the production of tPA activity following maturation is consistent with its ability to target the destruction of *Plat* mRNA (see Fig. 3A and Strickland et al., 1988), as well as its ability to inhibit translation of the *Plat* mRNA (Strickland et al., 1988). Nevertheless, injection of *Plat* sense RNA also modestly inhibited the production of tPA activity, although to

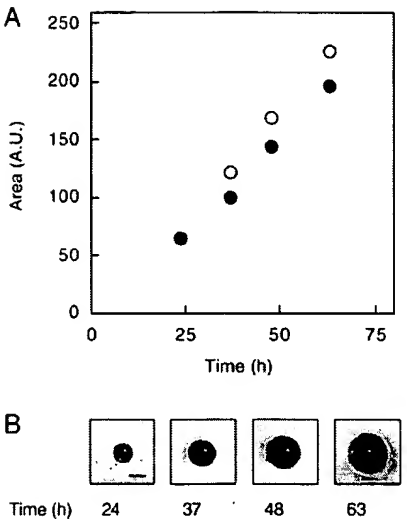

Fig. 7. Zymographic assay of tPA activity in single eggs. (A) Time-dependent increase in the area of the lytic zone of two eggs. (B) Photomicrographs of the lytic zone as a function of time of a single egg. Scale bar: 3 mm.

a lesser extent than either *Plat* antisense or dsRNA (Fig. 8, black bars). We observed that *Mos* sense RNA, which doesn't target the *Plat* mRNA, also resulted in a 50% decrease in tPA activity but had no inhibitory effect on the activation of MAP kinase (data not shown). The molecular basis underlying this inhibitory effect of sense RNA on the generation of tPA activity remains unresolved.

The results presented in Fig. 3 indicated that *Plat* dsRNA was more potent than *Plat* antisense RNA in targeting the reduction of endogenous *Plat* mRNA. As expected, a ten-fold dilution of *Plat* antisense RNA resulted in levels of tPA activity

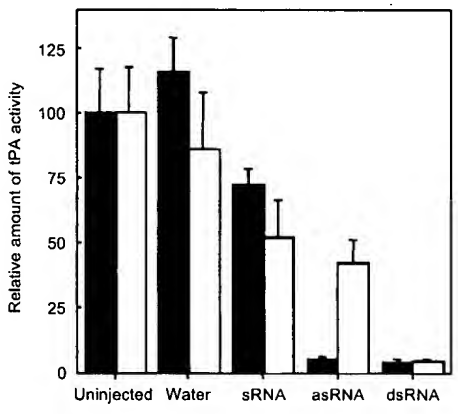

Fig. 8. Effect of *Plat* sense (s), antisense (as) and dsRNA (ds) on the appearance of tPA activity following oocyte maturation. Black bars, injection of approx. 10^6 molecules of RNA; white bars, injection of approx. 10^5 molecules of RNA. The value obtained in the uninjected oocytes was taken as 100% and the other samples are expressed relative to this amount. The data are expressed as the mean±s.e.m. and typically 5-12 eggs were assayed. The experiment was performed three times and similar results were obtained in each case; a representative experiment is shown.

similar to that of sense-injected oocytes, whereas injection of *Plat* dsRNA still promoted a dramatic inhibition (Fig. 8, white bars). Similar to the results obtained with *Mos* dsRNA, these results confirmed that *Plat* dsRNA was more potent than *Plat* antisense RNA in promoting the reduction of the endogenous *Plat* mRNA.

DISCUSSION

We have demonstrated that RNAi is an effective and efficient method to inhibit the translation of maternal mRNAs that are recruited during oocyte maturation. The reduction of the targeted mRNA, namely *Mos* and *Plat*, is specific, i.e., a nontargeted mRNA is not destroyed, and is both time- and concentration-dependent; mRNA levels can be decreased by up to 90%. In addition, dsRNA is more effective than antisense RNA. The reduction of *Mos* mRNA led to a failure in MAP kinase activation that normally accompanies oocyte maturation. A consequence of this failure is that metaphase II arrest was not maintained and that the eggs underwent parthenogenetic activation with the concomitant decrease in H1 kinase activity. Likewise, reduction of the endogenous *Plat* mRNA inhibited the production of tPA following oocyte maturation. A recent report also found that injection of *Mos* dsRNA results in parthenogenetic activation of mouse eggs (Wianny and Zernicka-Goetz, 2000); it was not shown in that study, however, that the *Mos* RNA is selectively degraded and that MAP kinase failed to activate. In the mouse, RNAi, which entails microinjection of the dsRNA, should prove far superior to antisense approaches that have been used in the past, but with variable success. It should be noted that culture of oocytes in medium containing either *Plat* or *Mos* dsRNA (2 μg/μl) does not reduce the amount of the targeted mRNA (P. S., P. S. and R. M. S., unpublished). Thus, in contrast to lower species such as *C. elegans* (Fire et al., 1998) and planaria (Sanchez-Alvarado and Newmark, 1999), in which injection of the dsRNA into the animal results in the reduction of the targeted mRNA, mouse oocytes apparently lack this uptake mechanism or, if it is present, it is very inefficient.

About 1.5×10^6 molecules of either *Plat* or *Mos* dsRNA were injected when undiluted dsRNA was used. This corresponds to an intracellular concentration of 10 nM, as the volume of an oocyte is approx. 250 pl. This concentration is similar to that required to ablate *frizzled* function in *Drosophila* embryos (Kennerdell and Carthew, 1998). In those experiments approx. 0.2 fmole of dsRNA was injected into syncytial blastoderm embryos whose volume is approx. 7.3 nl and this corresponds to approx. 25 nM. Concentrations of 10 nM dsRNA are effective in an in vitro system that supports the destruction of the targeted mRNA (Tuschl et al., 1999). Significant reduction of both *Mos* and *Plat* mRNAs are also observed when only 10,000 molecules of *Mos* or *Plat* dsRNA are injected. As the oocyte contains about 10,000 each of these transcripts (Huarte et al., 1987; Keshet et al., 1988), the reduction of the endogenous mRNA appears to be very efficient. A catalytic mechanism is possible, as in other systems the number of dsRNA molecules per cell is likely to be less than the number of endogenous transcripts. For example, in *C. elegans*, injection of 60,000 *unc*-22 dsRNA into adult animals results in the twitching phenotype in approx. 100 progeny (Fire et al.,

1998). *unc*-22 expression commences when the embryos contain about 500 cells, by which time the injected dsRNA would be diluted to only a few molecules per cell. Alternatively, the recent finding that an RNA-directed RNA polymerase is implicated in RNAi (Smardon et al., 2000) could provide an amplification mechanism that accounts for the efficacy of stoichiometric or substoichiometric amounts of dsRNA to promote the efficient reduction of the targeted mRNA.

When approx. 10^6 or 10^4 molecules of either *Mos* or *Plat* dsRNA are injected, the kinetics of *Plat* mRNA degradation are slower than that for *Mos* mRNA. As it has been estimated that oocytes contain approx. 10 000 transcripts of each of these mRNAs, the difference in kinetics of mRNA degradation may reflect that the *Mos* mRNA is more accessible to be targeted for destruction. It should be borne in mind, however, that estimate of the number of transcripts is relatively crude, and hence the difference in kinetics of mRNA degradation may reflect differences in transcript abundance, i.e., there is less *Mos* mRNA than *Plat* mRNA.

Both *Mos* and *Plat* antisense RNA are also effective in reducing the amount of endogenous *Mos* and *Plat* mRNA, respectively. Nevertheless, on a molar basis, the antisense RNA is not as effective as dsRNA. For example, *Mos* dsRNA more effectively inhibits the activation of MAP kinase when compared with *Mos* antisense RNA; parthenogenetic activation and the concomitant reduction in histone H1 kinase activity are only observed in *Mos* dsRNA-injected eggs, and not in *Mos* antisense RNA-injected eggs. This suggests in turn that MAP kinase activity must be reduced below a threshold level at which MAP kinase activity is almost absent, in order to make the eggs exit metaphase II arrest and enter interphase. Moreover, when the amount of injected *Mos* dsRNA and antisense RNA are reduced 10-fold, *Mos* dsRNA is still highly effective in inhibiting the increase in MAP kinase activity whereas *Mos* antisense RNA is essentially ineffective. Thus, *Mos* dsRNA is more efficient than *Mos* antisense RNA. A similar situation is also found with *Plat* antisense and dsRNA. Injection of 10^6 molecules of *Plat* antisense or dsRNA results in both destroying the *Plat* mRNA and inhibiting the increase in tPA activity that accompanies oocyte maturation. In contrast, injection of 10^5 molecules of *Plat* antisense RNA results in little reduction of the endogenous mRNA and little inhibition in the increase in tPA activity, while *Plat* dsRNA still results in the reduction of the endogenous mRNA and inhibition of the appearance of tPA activity.

The increased potency of dsRNA when compared with antisense RNA could, in principle, reflect differences in their stability, i.e., dsRNA is more stable than antisense RNA. This possibility is minimized by the observation that in a *Drosophila* cell lysate that supports RNAi-mediated mRNA destruction both capped antisense and capped dsRNA are stable but only the capped dsRNA is active (Tuschl et al., 1999). Moreover, results of recent experiments suggest that processing the dsRNA to discrete 20-25 nucleotide fragments is part of the mechanism that leads to destruction of the targeted mRNA (Hammond et al., 2000; Zamore et al., 2000). In fact, asRNA can give rise in an in vitro system to small amounts of stable 20-25 nucleotide fragments (Zamore et al., 2000). This could account for the activity, albeit reduced, of antisense RNA, relative to dsRNA.

dsRNAs in mammalian cells typically activate protein kinase PKR that phosphorylates and inactivates eIF2a (Fire, 1999). The ensuing inhibition of protein synthesis ultimately results in apoptosis. This sequence-independent response may reflect a form of primitive immune response, since the presence of dsRNA is a common feature of many viral lifecycles. Mouse oocytes, however, clearly lack this response, as oocyte maturation beyond germinal vesicle breakdown requires protein synthesis (Wassarman et al., 1976), which probably reflects a requirement for cyclin B synthesis, and the oocytes injected with dsRNAs resume meiosis and mature to metaphase II. Preimplantation mouse embryos also lack the response, as embryos injected with dsRNAs develop to the blastocyst stage (Wianny and Zernicka-Goetz, 2000). When the embryo acquires this response is unknown. It is not known if oocytes and preimplantation embryos contain PKR activity, which could account for the lack of the response. The lack of this response, however, cannot be attributed to a deficiency in the cell death machinery, because both oocytes (Perez et al., 1999) and preimplantation embryos (Brison and Schultz, 1997; Handyside and Hunter, 1986; Pierce et al., 1989; Weil et al., 1996) can undergo apoptosis.

The lack of this response to dsRNA may confer a selective advantage by minimizing reproductive wastage. Both oocytes and preimplantation embryos are exposed to viruses. Viral exposure throughout the lifespan of the female could deplete the pool of oocytes and compromise her reproductive capacity, because oocytes do not proliferate. The preimplantation embryo is also susceptible to viral infection from viruses present in the female reproductive tract. Infection of an early cleavage stage preimplantation embryo that results in blastomere death could result in a blastocyst containing an insufficient number of inner cell mass cells, and hence be incapable of development to term.

RNAi clearly offers several advantages to the current methods that employ generation of null mutants by homologous recombination, which requires (1) generating a suitable targeting construct, (2) selecting homologous recombination events in ES cells, (3) injecting blastocysts with these ES cells, and (4) establishing pure breeding lines from the chimeric offspring. The RNAi response will also likely be far more efficient and consistent than the antisense RNA approach that has been used with very inconsistent results in the mouse oocyte and embryo. Moreover, hypomorph phenotypes may become manifest, as RNAi does not appear to result in the total ablation of the targeted mRNA. Such hypomorph phenotypes may be as informative (or more informative) than the corresponding null mutation by providing novel insights into the presence of thresholds and/or the function of a gene. For example, as described above, a critical amount of MOS activity appears required for the development of a level of MAP kinase activity that is sufficient to maintain metaphase II arrest, a result consistent with a recently proposed switch mechanism for MAP kinase activation, as well as other cellular switches (Ferrell, 1999). In addition, modest changes in the levels of *Oct4* (*Pou5f1* – Mouse Genome Informatics) expression may also function as a developmental switch by regulating the fate of embryonic stem cells, e.g., high levels lead to differentiation into primitive endoderm and mesoderm, intermediate levels lead to pluripotent stem cells and reduced levels result in trophectoderm (Niwa et al., 2000).

As more dormant maternal mRNAs are identified, RNAi will be a valuable tool with which to study their function in oocyte maturation, fertilization and egg activation, and development. Moreover, the method can also be used to study the function of genes that are expressed in the early embryo, since dsRNA can inhibit the function of zygotically expressed genes (Wianny and Zernicka-Goetz, 2000; P. S., P. S. and R. M. S., unpublished). Whether dsRNA can also lead to DNA methylation of the targeted gene and result in long-term repression of transcription, as apparently occurs in plants (Wassenegger et al., 1994), is unknown.

This research was supported by a grant from the NIH (HD 22681 to R.M.S.). The authors would like to thank Johanna Mestach for help with preparing the dsRNA.

REFERENCES

Brison, D.R. and Schultz, R.M. (1997). Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor α. *Biol. Reprod.* **56**, 1088-1096.

Carroll, P.M., Richards, W.G., Darrow, A.L., Wells, J.M. and Strickland, S. (1993). Preimplantation mouse embryos express a cell surface receptor for tissue-plasminogen activator. *Development* **119**, 191-198.

Chatot, C.L., Ziomek, C.A., Bavister, B.D., Lewis, J.L. and Torres, I. (1989). An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *J. Reprod. Fertil.* **86**, 679-688.

Colledge, W.H., Carlton, M.B.L., Udy, G.B. and Evans. M.J. (1994). Disruption of c-mos causes parthenogenetic development of unfertilized mouse eggs. *Nature* **370**, 65-68.

Eppig, J.J. and O'Brien, M.J. (1996). Development in vitro of mouse oocytes from primordial follicles. *Biol. Reprod.* **54**, 197-207.

Ferrell, J.E. Jr (1999). Building a cellular switch: more lessons from a good egg. *BioEssays* **21**, 866-870.

Fire, A. (1999). RNA-triggered gene silencing. *Trends Genet.* **15**, 358-363.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-811.

Gebauer, F. and Richter, J.D. (1997). Synthesis and function of Mos: the control switch of vertebrate oocyte meiosis. *BioEssays* **19**, 23-28.

Hammond, S.M., Berstein, E., Beach, D. and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293-296.

Handyside, A.H. and Hunter, S. (1986). Cell division and death in the mouse blastocyst before implantation. *Roux's Arch. Dev. Biol.* **195**, 519-526.

Hasimoto, N., Watanabe, N., Y. Furuta, Y., H. Tamemoto, H., N. Sagata, N., M. Yokoyama, M., Okazaki, K., Nagayoshi, M., Takeda, N., Ikawa, Y. and Aizawa, S. (1994). Parthenogenetic activation of oocytes in c-*mos*-deficient mice. *Nature* **370**, 68-71.

Ho, Y., Wigglesworth, K., Eppig, J.J. and Schultz, R.M. (1995). Preimplantation development of mouse embryos in KSOM: Augmentation by amino acids and analysis of gene expression. *Mol. Reprod. Dev.* **41**, 232-238.

Huarte, J., Belin, D., Vassalli, A., Strickland, S. and Vassalli, J.-D. (1987). Meiotic maturation of mouse oocytes triggers the translation and polyadenylation of dormant tissue-type plasminogen activator mRNA. *Genes Dev.* **1**, 1201-1211.

Huarte, J., Belin, D. and Vassalli, J.-D. (1985). Plasminogen activator in mouse and rat oocytes: Induction during meiotic maturation. *Cell* **43**, 551-558.

Kennerdell, J.R. and Carthew, R.W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. *Cell* **95**, 1017-1026.

Keshet, E., Roenberg, M.P., Mercer, J.A., Propst, F., Vande Woude, G.F., Jenkins, N.A. and Copeland, N.G. (1988). Developmental regulation of ovarian-specific Mos expression. *Oncogene* **2**, 235-240.

Ketting, R.F., Haverkamp, T.H., van Luenen, H.G. and Plasterk, R.H. (1999). Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99**, 133-141.

Kurasawa, S., Schultz, R.M. and Kopf, G.S. (1989). Egg-induced modifications of the *zona pellucida* of mouse eggs: effects of microinjected inositol 1,4,5-trisphosphate. *Dev. Biol.* **133**, 295-304.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Latham, K.E., Doherty, A.S., Scott, C.D. and Schultz, R.M. (1994). Igf2r and Igf2 gene expression in androgenetic, gynogenetic, and parthenogenetic preimplantation mouse embryos: absence of regulation by genomic imprinting. *Genes Dev.* **8**, 290-299.

Li, Y.-X., Farrell, M.J., Liu, R., Mohanty, N. and Kirby, M.L. (2000). Double-stranded RNA injection produces null phenotypes in zebrafish. *Dev. Biol.* **217**, 394-405.

Lohmann, J.U., Endl, I. and Bosch, T.C.G. (1999). Silencing of developmental genes in Hydra. *Dev. Biol.* **214**, 211-214.

Manejwala, F.M., Logan, C.Y. and Schultz, R.M. (1991). Regulation of hsp70 mRNA levels during oocyte maturation and zygotic gene activation in the mouse. *Dev. Biol.* **144**, 301-308.

Misquitta, L. and Paterson, B.M. (1999). Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): A role for nautilus in embryonic somatic muscle formation. *Proc. Natl. Acad. Sci. USA* **96**, 1451-1456.

Montgomery, M.K., Xu, S. and Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. USA* **95**, 15502-15507.

Moos, J., Visconti, P.E., Moore, G.D., Schultz, R.M. and Kopf, G.S. (1995). Potential role of mitogen-activated protein kinase in pronuclear envelope assembly and disassembly following fertilization of mouse eggs. *Biol. Reprod.* **53**, 692-699.

Ngo, H., Tschudi, C., Gull, K. and Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in Trypanosoma brucei. *Proc. Natl. Acad. Sci. USA* **95**, 14687-92.

Nishida, E. and Gotoh, Y. (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* **18**, 128-131.

Niwa, H., Miyazaki, J. and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genet.* **24**, 372-376.

O'Keefe, S.J., Wolfes, H., Kiessling, A.A. and Cooper, G.M. (1989). Microinjection of antisense *c-mos* oligonucleotides prevents meiosis II in the maturing mouse egg. *Proc. Natl. Acad. Sci. USA* **86**, 7038-7042.

Paules, R.S., Buccione, R., Moschel, R.C., Vande Woude, G.F. and Eppig, J.J. (1989). Mouse *Mos* protooncogene product is present and functions during oogenesis. *Proc. Natl. Acad. Sci. USA* **86**, 5395-5399.

Perez, G.I., Tao, X.-J. and Tilly, J.L. (1999). Fragmentation and death (a.k.a. apoptosis) of ovulated oocytes. *Mol. Hum. Reprod.* **5**, 414-420.

Pierce, G.B., Lewellyn, A.L. and Parchment, R.E. (1989). Mechanism of programmed cell death in the blastocyst. *Proc. Natl. Acad. Sci. USA* **86**, 3654-3658.

Sagata, N. (1997). What does Mos do in oocytes and somatic cells? *BioEssays* **19**, 13-21.

Sanchez-Alvarado, A. and Newmark, P.A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci. USA* **96**, 5049-5054.

Schultz, R.M., Letourneau, G.E. and Wassarman, P.M. (1979). Program of early development in the mammal: Changes in patterns and absolute rates of tubulin and total protein synthesis during oogenesis and early embryogenesis in the mouse. *Dev. Biol.* **68**, 341-359.

Schultz, R.M., Montgomery, R.R. and Belanoff, J.R. (1983). Regulation of mouse oocyte maturation: implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. *Dev. Biol.* **97**, 264-273.

Schultz, R.M. and Wassarman, P.M. (1977). Specific changes in the pattern of protein synthesis during meiotic maturation of mammalian oocytes in vitro. *Proc. Natl. Acad. Sci. USA* **74**, 538-541.

Seeds, N.W., Basham, M.E. and Haffke, S.P. (1999). Neuronal migration is retarded in mice lacking the tissue plasminogen activator gene. *Proc. Natl. Acad. Sci. USA* **96**, 14118-14123.

Sharp, P.A. (1999). RNAi and double-strand RNA. *Genes Dev.* **13**, 139-141.

Smardon, A., Spoerke, J., Stacey, S., Klein, M., Mackin, N. and Maine, E. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr. Biol.* **10**, 169-178.

Sorensen, R.A. and Wassarman, P.M. (1976). Relationship between growth and meiotic maturation of the mouse oocyte. *Dev. Biol.* **50**, 531-536.

Strickland, S., Huarte, J., Belin, D., Vassalli, A., Rickles, R.J. and Vassalli, J.-D. (1988). Antisense RNA directed against the 3′ noncoding region prevents dormant mRNA activation in mouse oocytes. *Science* **241**, 680-684.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. and Mello, C.C. (1999). The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell* **99**, 123-132.

Temeles, G.L., Ram, P.T., Rothstein, J.L. and Schultz, R.M. (1994). Expression patterns of novel genes during mouse preimplantation embryogenesis. *Mol. Reprod. Dev.* **37**, 121-129.

Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P. and Sharp, P.A. (1999). Targeted mRNA degradation by double-stranded RNA *in vitro*. *Genes Dev.* **13**, 3191-3197.

Van Blerkom, J. (1981). Structural relationship and posttranslational modification of stage-specific proteins synthesized during early preimplantation development in the mouse. *Proc. Natl. Acad. Sci. USA* **78**, 7629-7633.

Vassalli, J.-D., Dayer, J.-M., Wohlwend, A. and Belin, D. (1984). Comcomitant secretion of prourokinase and of a plasminogen activator-specific inhibitor by cultured human monocytes-macrophages. *J. Exp. Med.* **159**, 1653-1668.

Vassalli, J.-D., Huarte, J., Belin, D., Gubler, P., Vassalli, A., O'Connell, M.L., Parton, L.A., Rickles, R.J. and Strickland, S. (1989). Regulated polyadenylation controls mRNA translation during meiotic maturation of mouse oocytes. *Genes Dev.* **3**, 2163-2171.

Verlhac, M.-H., De Pennart, H., Maro, B., Cobb, M.H. and Clarke, H.J. (1993). MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. *Dev. Biol.* **158**, 330-340.

Wargelius, A., Ellingsen, S. and Fjose, A. (1999). Double-stranded RNA induces specific developmental defects in zebrafish embryos. *Biochem. Biophys. Res. Commun.* **263**, 156-161.

Wassarman, P.M., Josefowicz, W.J. and Letourneau, G.E. (1976). Meiotic maturation of mouse oocytes in vitro: inhibition of maturation at specific stages of nuclear progression. *J. Cell Sci.* **22**, 531-545.

Wassenegger, M., Heimes, S., Riedel, L. and Sanger, H.L. (1994). RNA-directed de novo methylation of genomic sequences in plants. *Cell* **76**, 567-76.

Weil, W., Jacobson, M.D., Coles, H.S.R., Davies, T.J., Gardner, R.L., Raff, K.D. and Raff, M.C. (1996). Constitutive expression of the machinery for programmed cell death. *J. Cell Biol.* **133**, 1053-1059.

Wianny, F. and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. *Nat. Cell Biol.* **2**, 70-75.

Wickramasinghe, D., Ebert, K.M. and Albertini, D.F. (1991). Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes. *Dev. Biol.* **143**, 162-172.

Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000). RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33.

Zhao, X., Singh, B. and Batten, B.E. (1991). The role of c-mos proto-oncoprotein in mammalian meiotic maturation. *Oncogene* **6**, 43-49.

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